Role of Acetylcholinesterase in the Regulation of Mesenchymal Stem Cell Proliferation and Differentiation

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Abstract

Mesenchymal stem cells (MSCs) play a key role in the regulation of tissue maintenance and repair. These cells have multipotent potential *in vivo*, which can be maintained when they are cultured *in vitro*. MSCs can be derived from various tissues including bone marrow, umbilical cord, Wharton's jelly of the umbilical cord, placenta, adipose tissue, dental pulp, as well as from the lungs of lung transplant patients. *In vitro*, these cells are adherent to tissue culture flasks, display a fibroblast-like morphology, and maintain the ability to differentiate into osteoblasts, adipocytes, and chondroblasts. It has been demonstrated that MSCs are also capable of differentiating into cell types of different germ layer origins including neurons, alveolar epithelium, hepatocytes, and myocytes.

Currently, only a few signaling pathways involved in the regulation of MSC proliferation and differentiation have been identified (e.g. Wnt signaling pathway). Disruption of these pathways drastically alters the ability of MSCs to proliferate and differentiate, therefore, altering the ability of MSCs to participate in tissue maintenance and/or repair. Service members have the potential to be exposed to many different toxic chemicals. These chemicals could disrupt the normal functions of MSCs and interrupt the normal healing processes.

Recently, it has been reported that bone marrow-derived MSCs express active acetylcholinesterase (AChE) and that disruption of this activity by organophosphate chemicals affects the ability of MSCs to differentiate into osteoblasts. The role AChE plays in MSC proliferation and differentiation is currently unknown. In this study, we are studying the effects of organophosphates on AChE activity, proliferation, and differentiation of MSCs.

In the current study, we have established MSC toxicity profiles of the organophosphate chemical parathion (Pt) and its metabolite, paraoxon (Px), through the evaluation of cellular viability and proliferation. Our results indicate that Pt/Px affect cellular viability and proliferation in a dose- and time-dependent manner. We also demonstrated that treatment with Pt/Px reduce both AChE protein expression levels and activity in these cells in a dose-dependent manner, and determined the optimal Pt/Px doses required for AChE inhibition. Based on these data, we then determined if Pt/Px affected MSC differentiation into osteoblasts, adipocytes, chondrocytes, and neurons over several time courses. Prior treatment with Pt/Px significantly reduces the MSCs' ability to differentiate into adipocytes. These results suggest that Pt/Px are disrupting the normal pathways associated with AChE in human MSCs. Understanding the pathways associated with MSC proliferation and differentiation could lead to the development of future MSC-based tissue repair therapies.

Materials and Methods

Experimental Chemicals: Stock solutions of the organophosphate pesticides (OPP) parathion and paraoxon were prepared in 100% ethanol (EtOH). The cholinesterase reactivator pralidoxime (2-PAM) was prepared in sterile deionized water and stored at 4ºC

Human MSC Culture: Primary human bone marrow-derived MSCs were obtained from Lonza (Walkersville, MD) and cultured in Mesenchymal Stem Cell Growth Medium. Only MSCs from passages 4-8 were used.

Parathion/Paraoxon Toxicity Studies: We plated $1x10^4$ MSCs in the wells of 96-well tissue culture plates and allowed the cells to attach for 24 hours. MSCs were then exposed to increasing concentrations of parathion, paraoxon, or EtOH for 48 hours. A MTT Cell Viability Assay was then performed according to the manufacturer's protocol. Results were read on a SpectraMax® Plate Reader and expressed as % relative viability. For AChE reactivation studies, MSCs were exposed to media alone, EtOH, 2-PAM, Paraoxon or Paraoxon+2-PAM for 24 hours. The cells were then evaluated as stated.

MSC Proliferation Studies: To evaluate the effects of parathion/paraoxon on MSC proliferation, we plated $5x10^3$ MSCs in the wells of 96-well tissue culture plates and allowed the cells to attach for 24 hours. Next, we exposed the MSCs to increasing concentrations of parathion, paraoxon, or EtOH for 48 hours. MSC proliferation was then evaluated using the BrdU Cell Proliferation Assay according to the manufacturer. Plates were then read on a SpectraMax® Plate Reader and results expressed as % BrdU incorporation.

Determination of AChE Activity: AChE activity within the MSCs was measured using the colorimetric AChE Assay Kit according to the manufacturer's protocol.

Determination of AChE Expression in MSCs (Western blotting): Confluent MSCs were exposed to parathion, paraoxon, or vehicle (EtOH) for 24hours. Then, the MSCs were lysed and collected in 1X RIPA buffer, centrifuged, and the supernatants assayed for protein concentration with the Pierce™ 660nm Protein Assay. The samples were resolved using the 4-12% gradient Bolt™ Bis-Tris Plus Gel according to the manufacturer's instructions and transferred onto nitrocellulose membranes using the iBlot® 7-Minute Blotting System. The membranes were blocked, incubated with rabbit anti-AChE antibodies, and incubated with anti-rabbit IgG antibodies conjugated to alkaline phosphatase using the iBlot® Western Blot System according to the manufacturer's instructions. Finally, the membranes were developed using the iBlot® Western Detection, Chromogenic Kit. To ensure equal loading, equivalent samples were run and blots probed with anti-GAPDH antibodies and processed as stated above.

Adipogenic Differentiation of MSCs: MSCs were plated in 96-well tissue culture plates at a density of $5x10^3$ cells/well and allowed to grow to confluence. The media was replaced with Human MSC adipogenic induction medium and adipogenic maintenance medium alone or with parathion, paraoxon, or EtOH in cycles in accordance with the manufacturer's protocol, and cultured for 7-21 days. The cells were stained using the AdipoRed™ Assay Reagent protocol and measured using a microplate fluorescence spectrophotometer. Results were expressed as relative fluorescence units (RFU). MSCs were also evaluated for adipogenic differentiation by staining with Oil Red O followed by image analysis using ImageJ software (NIH).

Osteogenic Differentiation of MSCs: MSCs were plated in 96-well tissue culture plates at a density of $1-4x10^3$ cells/well. After attachment, but prior to confluence (24-72 hours), the media was replaced with osteogenic differentiation medium alone or with parathion, paraoxon, or vehicle (EtOH). This medium was replaced every 3-4 days for 7-21 days. Cells were fixed with absolute EtOH or 4% paraformaldehyde (PFA) for 20 minutes then stained using the OsteoImage™ Mineralization Assay protocol and measured using a microplate fluorescence spectrophotometer. Results were expressed as RFU.

Statistical Analysis: Two-way ANOVA was used to compare the mean responses among experimental and control groups. The Dunnett and Scheffe F-test was used to determine between which groups significant differences existed. A p-value of < 0.05 was considered significant for all experiments.

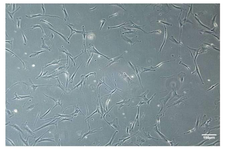

Figure 1: Cultured human MSCs. *A representative phase contrast image of undifferentiated human MSCs. Bar = 100µm.*

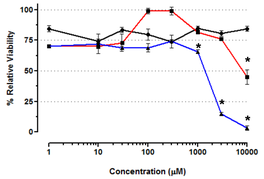

Figure 2: Effects of parathion and paraoxon on the viability of cultured human MSCs. *Human MSCs were exposed to increasing concentrations of parathion (red, ■), paraoxon (blue, ▲), or EtOH, (black, ●) for 48 hours, after which they were assayed for viability. The results are reported as mean ± standard error of the mean (SEM) of % Relative Viability; n ≥ 4 for each condition tested. *p ≤ 0.05 versus vehicle control.*

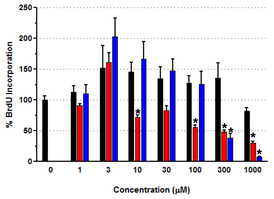

Figure 3: Effects of parathion and paraoxon on the proliferative ability of cultured human MSCs. *Human MSCs were exposed to increasing concentrations of parathion (red), paraoxon (blue), or EtOH, (black) for 48 hours. The cells were then assayed for cellular growth. The results are reported as mean ± SEM of % BrdU Incorporation; n ≥ 4 for each condition tested. *p ≤ 0.05 versus vehicle control.*

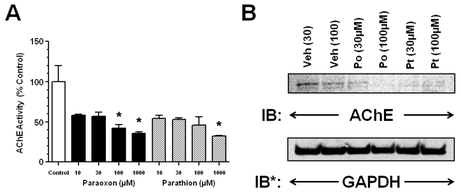

Figure 4: Effects of parathion and paraoxon on the activity and expression of AChE in human MSCs. *A: MSCs were exposed to increasing concentrations of paraoxon (filled), parathion (cross-hatched), or media alone (open) for 24 hours. The cells were then assayed for AChE activity. Vertical bars represent mean ± SEM of AChE Activity (percent control); n ≥ 2 for each condition tested. *p ≤ 0.05 versus media control. B: Human MSCs were exposed to paraoxon, parathion, or EtOH for 24 hours and blotted for AChE. IB: immunoblot. IB*: immunoblot of housekeeping gene product. The blot is representative of 3 independent experiments.*

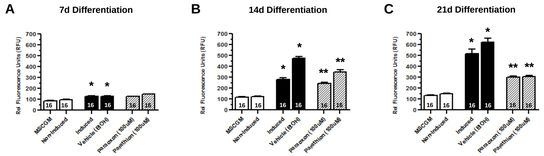

Figure 5: Evaluation of OPPs on MSC adipogenic differentiation. *Quantitative graph of RFU for each adipogenic differentiation condition. MSCs were exposed to adipogenic differentiation media for (A) 7, (B) 14, or (C) 21 days and then evaluated for differentiation using the fluorescence-based AdipoRed Assay Reagent. The results are reported as mean ± SEM of RFU. Open bars represent negative control conditions; filled bars represent positive control conditions; cross-hatched bars represent experimental conditions. n for each condition is indicated within each bar. *p < 0.05 versus non-induced control; **p < 0.05 versus vehicle control.*

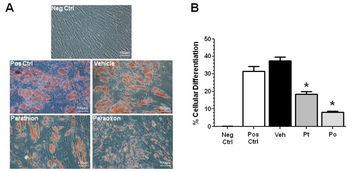

Figure 6: Human MSC adipogenic differentiation. *A: Representative phase contrast images of Oil Red O stained adipogenic differentiated MSCs. Bar = 100µm. B: Analysis of photomicrographs of adipogenic differentiation using Image J software. Vertical bars represent the mean ± SEM of percent cellular differentiation; *p ≤ 0.05 versus positive control.*

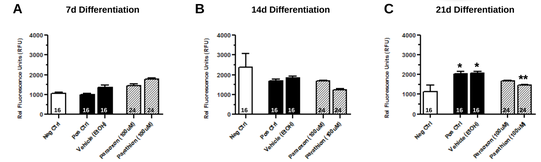

Figure 7: Evaluation of OPPs on MSC osteogenic differentiation. *Quantitative graph of RFU for each osteogenic differentiation condition. MSCs were exposed to osteogenic differentiation media for (A) 7, (B) 14, or (C) 21 days and then evaluated using the OsteoImage Mineralization Assay. The results are reported as mean ± SEM of RFU. Open bars represent negative control conditions; filled bars represent positive control conditions; cross-hatched bars represent experimental conditions. n for each condition is indicated within each bar. *p < 0.05 versus negative control; **p < 0.05 versus vehicle control.*

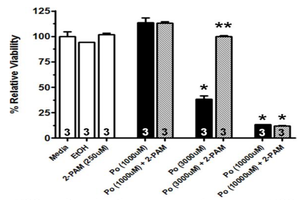

Figure 8: Effects of 2-PAM on MSCs cultured in the presence of paraoxon. *MSCs were exposed to media alone, EtOH, 2-PAM, increasing concentrations of paraoxon, or increasing concentrations of paraoxon + 2-PAM (250µM) for 24 hours, after which they were assayed for viability using the MTT Cell Viability Assay. Open bars indicate negative controls; filled bars represent exposure to paraoxon; cross-hatched bars indicate exposure to paraoxon + 2-PAM. The results are reported as mean ± SEM of percent Relative Viability; n = 3 for each condition tested. *p < 0.05 versus vehicle control; **p < 0.05 versus paraoxon.*

Conclusions

Our results indicate that the organophosphate chemicals, parathion and paraoxon, affect the viability, proliferative ability and differentiation potential of human bone marrow-derived MSCs. Here, we demonstrated that both parathion and paraoxon reduced the cellular viability of hMSCs (Fig 2). The minimum effective concentration was 1000µM for paraoxon and 10000µM for parathion following a 48-hour exposure. The IC_{50} for organophosphate-induced cell death was 1961µM for paraoxon and >10000µM for parathion. Lower concentrations of these chemicals reduced the proliferative potential of hMSCs at doses of ≥ 100µM (parathion) and ≥ 300µM (paraoxon) following a 48-hour exposure (Fig 3); the concentrations tested that reduced hMSC proliferation were not associated with increased cellular death. We also demonstrated that treatment with parathion and paraoxon reduced both AChE protein expression levels (Fig 4B) as well as activity (Fig 4A). These results suggest that parathion and paraoxon are disrupting the normal pathways associated with AChE in hMSCs; these altered pathways are inducing cellular death as well as disrupting the normal proliferative function of MSCs. We have also demonstrated the ability to differentiate hMSCs into different cell types including adipocytes (Figs 5-6) and osteoblasts (Fig 7). Prior treatment with either parathion or paraoxon reduces the hMSCs' ability to differentiate into adipocytes (Figs 5-6) or osteoblasts (Fig 7). Finally, we have demonstrated that treatment of hMSCs with an AChE reactivator (2-PAM) can rescue the cells from paraoxon-induced cellular death (Fig 8). Together, our results suggest that AChE is, at least in part, responsible for several MSC pathways including viability, growth, and differentiation.

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